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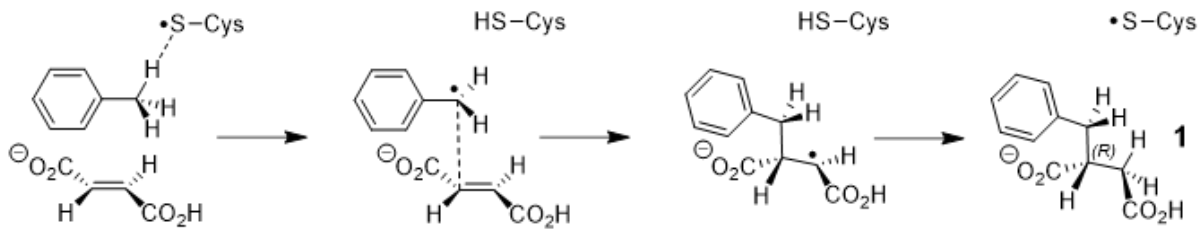
Stereochemistry of enzymatic benzylsuccinate synthesis elucidated with chirally labeled toluenes

Deniz Seyhan, Peter Friedrich, Maciej Szaleniec, Markus Hilberg, Wolfgang Buckel, Bernard T. Golding and Johann Heider*

Abstract: Benzylsuccinate synthase is a glycyl radical enzyme initiating anaerobic toluene metabolism by adding fumarate to the methyl group of toluene to yield (*R*)-benzylsuccinate. To investigate whether the reaction occurs with retention or inversion of configuration at the methyl group of toluene, we synthesized both enantiomers of chiral toluene, containing all three H isotopes in their methyl groups. The chiral toluenes were converted to benzylsuccinates containing preferentially ^{2}H and ^{3}H at their benzylic C-atoms, owing to a kinetic isotope effect favoring hydrogen abstraction from the methyl groups. The configuration of the products was analyzed by enzymatic CoA-thioester synthesis and stereospecific oxidation using enzymes involved in benzylsuccinate degradation. Assessment of the configurations of the benzylsuccinate isomers via loss or retention of tritium showed that inversion of configuration at the methyl group occurs when the chiral toluenes react with fumarate.

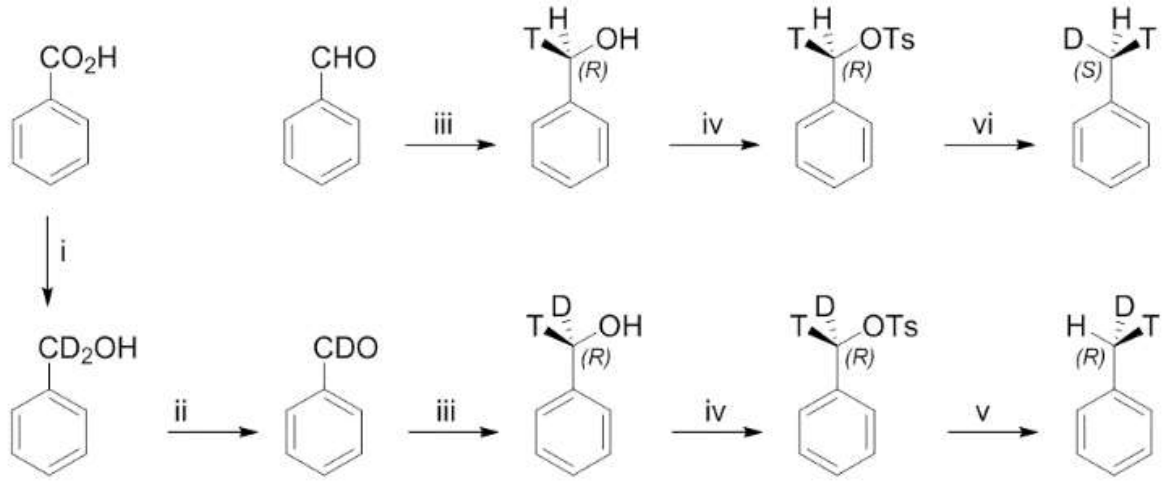
The glycyl radical enzyme benzylsuccinate synthase (BSS) replaces dioxygen-dependent mono- or di-oxygenases in initiating bacterial toluene degradation under anaerobic conditions. It represents one of many enzymes characterized in recent years, which use radical mechanisms to catalyze chemically demanding reactions in biochemistry, presenting a model case how radical-based reactions can still be performed stereospecifically.[1] BSS is activated to the radical state by a special activating enzyme which belongs to the 'radical SAM' family and requires S-adenosylmethionine for converting a conserved glycine within the catalytic subunit of BSS to a glycyl radical. Activated BSS then catalyzes radical addition of a fumarate co-substrate to the methyl group of toluene, leading to the stereospecific synthesis of (*R*)-benzylsuccinate (**1**, see Scheme 1).[1] The reaction pathway starts with a hydrogen atom transfer from a conserved active-site cysteine to the glycyl radical, generating a reactive thiyl radical. With BSS, the thiyl radical is proposed to abstract a hydrogen atom from the methyl group of toluene, forming an enzyme-bound benzyl radical as intermediate, which is poised to undergo addition to the *Re-Re* face of the double bond of a bound fumarate, yielding an intermediate (*R*)-benzylsuccinyl radical. This radical is quenched by hydrogen transfer from the active-site cysteine, and after re-establishing the stable glycyl radical state of BSS, the product is released and new substrates are bound,[2] (Scheme 1). In accordance with the proposed mechanism, the initially abstracted hydrogen atom is returned to the intermediate radical in a *syn*-addition mode.[3]



Scheme 1. Proposed BSS-mechanism.

In recent years, BSS has become a paradigm for many paralogous fumarate-adding enzymes for aromatic or aliphatic hydrocarbons.[4] In this study, we explore the 'cryptic stereochemistry'[5] of BSS towards the methyl group of toluene, i.e. does the reaction lead to retention or inversion of configuration at this position? This kind of study was pioneered in 1969 with chiral acetates containing all three H isotopes in their methyl groups.[6] Here, we synthesized both enantiomers of chiral toluene and converted them to benzylsuccinate with ^{14}C-labeled fumarate. The respective stereochemical configurations of the benzylsuccinate products were analyzed by converting them with the next two enzymes of toluene degradation, benzylsuccinate CoA-transferase (BS-CT) and benzylsuccinyl-CoA dehydrogenase (BS-DH), which results in either retention or loss of the ^{3}H-label.

Our experiments depended on a strong primary kinetic isotope effect (KIE) for the action of BSS on the chiral toluenes with preferential removal of the (^{1}H)-substituent and enrichment of stereochemically defined [1-^{3}H],(1-^{2}H)-benzylsuccinates. As KIE values were previously only reported from a different BSS isoenzyme from *Thauera aromatica* strain T1,[7] we determined the KIE of BSS from *T. aromatica* strain K172, which was used in this study. Specific BSS activities of 4.0 and 16 nmol min^{-1} (mg protein)$^{-1}$ were measured with ($^{2}H_8$)-toluene and unlabeled toluene, respectively, indicating a KIE value of 4.0. Control assays with unlabeled toluene and (2,3-$^{2}H_2$)fumarate yielded a specific activity of 18 nmol min^{-1} (mg protein)$^{-1}$. Therefore, we used a KIE value of 4.0 between ^{1}H and ^{2}H for our further calculations, rather than the somewhat lower values of 2.9 to 3.1 reported for BSS of strain T1.[7] According to the Swain-Schaad equation, this corresponds to a KIE value of 7.4 between ^{1}H and ^{3}H.[8] This rather high intermolecular KIE should provide a reliable basis to obtain significant differences between the two toluene enantiomers, although the experiments actually depend on the intramolecular KIE values of the chiral toluenes, which are not available and may deviate slightly from the intermolecular KIE values[9] (see also supplemental information for QM-based KIE estimates).



Scheme 2. Synthesis of chiral toluenes. Reagents and solvents: i, $LiAlD_4$/diethyl ether; ii, MnO_2/CH_2Cl_2; iii, yeast alcohol dehydrogenase and formate dehydrogenase/[^{3}H]formate/NAD^+/aq. phosphate buffer (pH 7.0) under argon; iv, p-toluenesulfonyl chloride/K_2CO_3; v, $LiAlH_4$/tetraglyme; vi, $LiAlD_4$/tetraglyme (detailed reaction conditions in supplemental information).

(*R*)- and (*S*)-[1-$^{3}H_1$],(1-$^{2}H_1$,1-$^{1}H_1$)toluene (called (*R*)- and (*S*)-toluene henceforth) were synthesized via a sequence of chemical and enzymatic reactions (Scheme 2). (1-$^{2}H_1$)benzaldehyde, the starting material for (*R*)-toluene was synthesized by reducing benzoic acid to dideuterated benzyl alcohol,

followed by MnO_2-mediated oxidation, resulting in (1-$^2H_1$)benzaldehyde containing a high ^2H content (99.3%) with a yield of 70.5% relative to benzoic acid (see supplemental information). To obtain both enantiomers of chiral toluenes, we set up two parallel routines starting with either unlabeled benzaldehyde or (1-$^2H_1$)benzaldehyde, which were converted to (*S*)- or (*R*)-toluene, respectively, in a three-step reaction sequence. The first reaction was stereoselective reduction by [^3H]NADH by yeast alcohol dehydrogenase,[10] resulting in *Re*-addition of ^3H to the benzaldehyde isotopomers. [^3H]Formate and formate dehydrogenase from *Candida boidinii* were used to generate [^3H]NADH in situ (see supporting information). This procedure yielded the (*R*)-enantiomers of [1-^3H],(1-^1H) and [1-^3H],(1-^2H)benzyl alcohol, respectively, with yields of 72%. After this step, the isotopically labeled benzyl alcohols were diluted with unlabeled benzyl alcohol, which does not interfere with the planned experiments, because the ^3H-labeled chiral centres necessary to evaluate the results were already established. The labeled benzyl alcohols were activated to the corresponding tosylates, which were reduced with inversion of configuration[11]to the desired chiral toluenes by using either $LiAlH_4$ (for (*R*)-toluene) or $LiAl(^2H)_4$ as reductant (for (*S*)-toluene; see Scheme 2). The final yields were 0.51 g (5.4 mmol) of (*S*)-toluene containing 32.2 kBq of radioactivity (specific activity 6.0 kBq/mmol), and 0.45 g (4.8 mmol) of (*R*)-toluene containing 38.9 kBq of radioactivity (specific activity 8.1 kBq/mmol), representing about 24% relative to the starting amounts of the benzaldehyde isotopomers. The amounts and specific activities of the obtained chiral toluenes were sufficient to distinguish between retention and inversion.

The chiral toluene enantiomers (100 Bq) were converted to benzylsuccinate by incubating them anaerobically for 16 h with added ^{14}C-labeled fumarate (33 Bq) and extracts of toluene-grown *T. aromatica* cells at room temperature as described previously.[12] The assays were then adjusted to pH 1.5 by adding aqueous HCl and the benzylsuccinate formed was removed by solid-phase extraction using a silica-based C18 column. The specific activities of BSS in the various replicate assays were determined as 10 to 20 nmol min^{-1} (mg protein)$^{-1}$, and the yields of benzylsuccinate in the conversion assays were between 0.32 to 0.54 µmol. These products (**2a/2b**) are expected to contain mainly the ^2H - and ^3H- atoms of the former methyl group of toluene (Figure 1), and their contents of ^3H and ^{14}C were determined by scintillation counting. The relative retention or loss of ^3H during the further conversion reactions (Figure 1) was based on their $^3H/^{14}C$ ratios.

To evaluate their configurations, the extracted benzylsuccinates (**2a/2b**) were converted along the pathway of benzylsuccinate □-oxidation by reacting them with purified BS-CT[13] and BS-DH[14a] (Figure 1A) giving via CoA-thioesters **3a/3b** the benzylidenesuccinyl-CoA thioesters **4a/4b**. To achieve this, we used BS-CT from the toluene-degrading species *Geobacter metallireducens* mixed with BS-DH from *T. aromatica*. The extracted doubly-labeled benzylsuccinates (**2a/2b**) from conversion of the chirally labeled toluenes were measured for their initial contents of ^3H and ^{14}C by scintillation counting and incubated with the two auxiliary enzymes under conditions favoring their complete conversion to benzylidenesuccinyl-CoA (**4a/4b**; see supplemental information). The reaction was complete after 3 h, as confirmed by HPLC analysis (data not shown). The solutions were acidified with HCl to pH 1.5 and the organic acid products obtained by solid-phase extraction were again analyzed for their ^3H and ^{14}C content. The results revealed that 75% of the ^3H content of **2b** derived from (*R*)-toluene was lost in the corresponding intermediate **4b**, whereas **4a** derived from (*S*)-toluene retained 79% of the ^3H content (Figure 1B). As BS-DH catalyzes an *anti* elimination of the hydrogen atoms at C2 and C3,[14] this demonstrates that the configuration of the methyl group of toluene is inverted during benzylsuccinate synthesis. Using KIE values of 4.0 and 7.4 for $^1H/^2H$ and $^1H/^3H$, respectively, the initial attack of BSS should occur at 72% of the ^1H, 18% of the ^2H and 10% of the ^3H substituents.

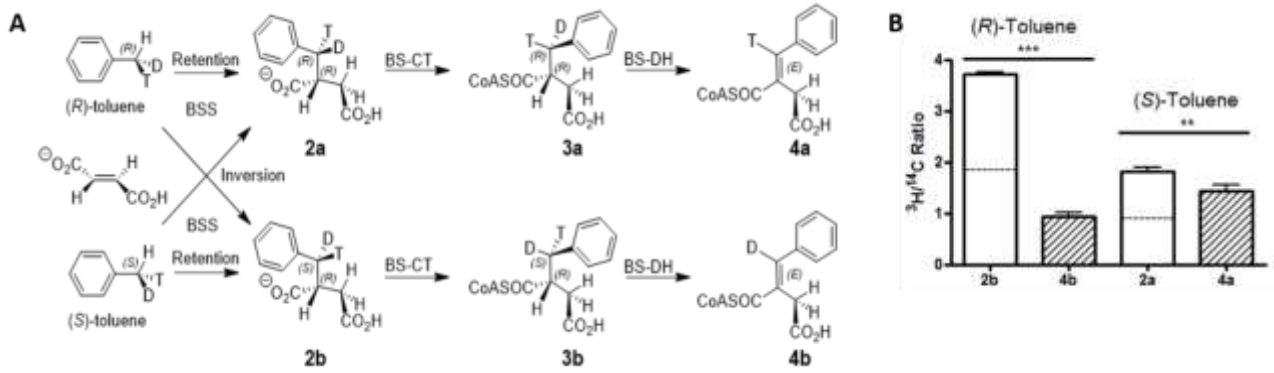


Figure 1. Configurational analysis of benzylsuccinate generated from chirally labeled toluene. **A**. Possible results of benzylsuccinate synthesis with chiral toluene and the further enzymatic conversion reactions. **B**. $^3H/^{14}C$ ratios of benzylsuccinate and benzylidenesuccinyl-CoA derived from chiral toluenes. Mean values and standard errors are from four independent experiments performed with three parallel measurements each. Mean retention values of tritium in benzylidenesuccinyl-CoA are 25% for (*R*) - and 79% for (*S*)- toluene. The dotted lines show the expected values in the respective products in a non-stereoselective reaction or with racemic **2a/2b**. Total radioactivity in the analyzed samples varied from 12 - 18 Bq ^3H and 2 - 5 Bq ^{14}C with (*R*)-toluene and from 13 - 21 Bq ^3H and 2 - 12 Bq ^{14}C with (*S*)-toluene.

Any initially abstracted ^3H will be re-donated to benzylsuccinate at a position not affected by BS-DH, resulting in expected ^3H-retention values in benzylidenesuccinyl-CoA of 82% (**4a**) and 28% (**4b**) for pure (*S*)- and (*R*)-toluene, respectively. The experimentally observed values of 79% and 25% (Figure 1B) are fully consistent with the expected values for enantiomer purities of the chiral toluenes of >90% (see supplemental information). The mean $^3H/^{14}C$ ratios of both pairs of the respective benzylsuccinate and benzylidenesuccinyl-CoA intermediates (**2a** and **4a** vs. **2b** and **4b**) were found to be significantly different ($P<0.05$) by paired two-tailed t-tests (Figure 1B).

The same stereospecificity of fumarate addition to toluene was predicted by the recently solved X-ray structure of the enzyme containing both substrates and by a quantum mechanics (QM) modeling approach on the BSS reaction mechanism[15] (see supporting information; Figures S1, S2). The observed result is also consistent with a recent report on methylpentylsuccinate synthase, a paralogue of BSS which is involved in anaerobic alkane degradation and adds fumarate to the C2 methylene group of n-hexane.[4c] Using chirally labelled hexane containing ^1H and ^2H at the C-2 and C-5 positions, the resulting methylpentylsuccinate-adduct was shown to originate with inversion of the configuration at C-2.[16]

Acknowledgements

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Keywords: benzylsuccinate synthase • chiral methyl group • toluene • glycyl radical • stereochemistry

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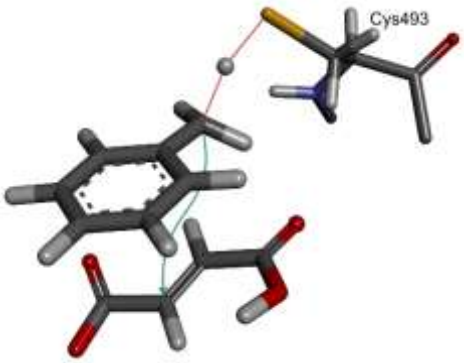
Stereochemistry of enzymatic benzylsuccinate synthesis with chirally labeled toluenes

Deniz Seyhan, Peter Friedrich, Maciej Szaleniec, Markus Hilberg, Wolfgang Buckel, Bernard T. Golding, Johann Heider*



Attack from the backside. Radical-based enzymatic addition of fumarate to the methyl group of toluene by benzylsuccinate synthase occurs with inversion of configuration as shown experimentally with both enantiomers of chiral toluene. This result is consistent with the predicted structure of a modelled transition state complex for initial hydrogen abstraction from toluene.

Supplemental information

Material and Methods

Synthesis of chiral toluenes

The syntheses of chiral toluenes [(*R*)- and (*S*)-toluene] described below were based on prior model experiments in which (*Me*-$^2H_1$)toluene and (*Me*-$^2H_2$)toluene were synthesised from benzyl alcohol and benzoic acid, respectively. Unlabelled benzyl alcohol was converted into the corresponding *p*-toluenesulfonate using a solvent-free method in which the alcohol was ground with potassium carbonate and *p*-toluenesulfonyl chloride.[1] The tosylate was reduced with $LiAl(^2H)_4$ in tetraglyme to give (*Me*-$^2H_1$)toluene, which was distilled directly from the reaction mixture. Reduction of benzoic acid with $LiAl(^2H)_4$ in diethyl ether gave (1-$^2H_2$)benzyl alcohol (99% ^{2}H-content by ^{1}H NMR). The dideutero-alcohol was converted as before into its tosylate, which was reduced with $LiAlH_4$ in diethyl ether to give (*Me*-$^2H_2$)toluene.

NMR data for (*Me*-$^2H_1$)toluene:

δ_H ($CDCl_3$, 400 MHz) 2.36 (br s, 2H, C*H*$_2$D), 7.1-7.4 (m, 5H, ArH); δ_C ($CDCl_3$, 100 MHz) 21.31 (1:1:1 t, ^1J ^{13}C-D 19.2 Hz, *C*H$_2$D), 125.42 (C-4), 128.35 and 129.16 (C-2, C-3, C-5, C-6), 137.96 (C-1).

NMR data for (*Me*-$^2H_2$)toluene:

δ_H ($CDCl_3$, 400 MHz) 2.40 (br s, 1H, C*H*D$_2$), 7.2-7.4 (m, 5H, ArH); δ_C ($CDCl_3$, 100 MHz) 21.08 (1:2:3:2 1 pentuplet, ^1J ^{13}C-D 19.2 Hz, *C*HD$_2$), 125.49 (C-4), 128.40 and 129.21 (C-2, C-3, C-5, C-6), 137.96 (C-1).

In a similar manner, but with the inclusion of an enzymatic reduction step successfully trialed with unlabeled materials throughout, (*R*) - and (*S*)-toluene were synthesized from benzaldehyde and (1-^{2}H)-benzaldehyde, respectively. (1-^{2}H)Benzaldehyde was synthesized from [1-$^2H_2$]benzyl alcohol (see above) by oxidation with MnO_2 [2] (Fig. 2). A benzaldehyde isotopomer (14 mM) was solved in 100 mM K-phosphate buffer (pH 7.0) and NAD^+ was added (final concentration 0.24 mM). The solutions were bubbled with argon for 20 min and transferred into an anaerobic chamber under an atmosphere of 95 % N_2 and 5 % H_2. Stereospecific enzymatic reduction[3] was performed with yeast alcohol dehydrogenase (3 U/50 ml), formate dehydrogenase from *Candida boidinii* (5 U/50 ml), and ^{3}H-labelled sodium formate (18.8 mM, specific activity of 2.9 GBq/mol). The reaction was stirred for 4 h at room temperature, then further portions of the enzymes were added and the incubations continued for 3 d. The mixture was extracted twice with diethyl ether. To ensure complete extraction of the labeled product and to generate enough starting material for the following reactions, unlabeled benzyl alcohol

was added (0.5 mL), followed by three more extraction steps. The extracts were dried over $MgSO_4$, filtered and evaporated. To generate the necessary amount of material for the subsequent syntheses, the product was further diluted with unlabeled benzyl alcohol (1.5 mL). Owing to the stereospecificity of the alcohol dehydrogenase, the method used gave specifically the (*R*)-enantiomers of [1-^{3}H],(1-^{1}H)benzyl alcohol (1.88 g = 17.4 mmol containing 380 kBq of radioactivity) and [1-^{3}H,1-^{2}H]benzyl alcohol (1.92 g = 17.7 mmol containing 170 kBq of radioactivity), respectively. The benzyl alcohol isomers obtained were converted into the corresponding tosylate by mixing the alcohol in a mortar with dry K_2CO_3 (10.0 g, 72.5 mmol) and *p*-toluenesulfonyl chloride (5.72 g, 30 mmol), followed by vigorous grinding for 30 min. Remaining *p*-toluenesulfonyl chloride was removed by adding powdered KOH (5.6 g, 100 mmol) and grinding for 15 min. The tosylate was extracted from the solid mixture by repeated diethyl ether addition and filtration. After evaporation of the ether under reduced pressure, the tosylate was obtained as a white solid.[1]

(*R*)-[1-^{3}H],(1-^{1}H)benzyl alcohol tosylate: 3.14 g (12 mmol; 67%).

(*R*)-[1-^{3}H],(1-^{2}H)benzyl alcohol tosylate: 1.88 g (11.4 mmol; 66%).

A tosylate was dissolved (~ 1.5 M) in tetraethyleneglycol dimethyl ether (tetraglyme, bp 275 ^{o}C), cooled to 0 °C in an ice bath and carefully mixed with a slight excess of either $LiAl(^2H)_4$ (0.55 g, 13 mmol, for the (*R*)-[1-^{3}H],(1-^{1}H)-tosylate) or $LiAlH_4$ (0.50 g, 13 mmol, for the (*R*)-[1-^{3}H],(1-^{2}H)-tosylate, Fig. 2). The reactions were allowed to come to room temperature and heated under reduced pressure at 50 to 60 °C, resulting in selective distillation of an enantiomer of chirally labelled toluene.

(*S*)-toluene from the reaction with $LiAl(^2H)_4$: 0.51 g = 5.4 mmol containing 32.2 kBq ^{3}H, specific activity 6.0 kBq/mmol).

(*R*)-toluene from the reaction with $LiAlH_4$: 0.45 g = 4.8 mmol containing 38.9 kBq ^{3}H, specific activity 8.1 kBq/mmol).

Preparation of cell free extract from *Thauera aromatica* K172 cells and conversion of toluene to benzylsuccinate

Thauera aromatica was grown anaerobically on toluene to an OD of 3 - 4 in 2L-bottles as described.[4] The cells were harvested under anaerobic conditions via centrifugation at 6,000 × g at 4 °C for 10 min. The cell pellets (ca. 15 - 17 g wet mass) were re-suspended in 15 - 17 mL 10 mM triethanolamine pH 7.5. The cell suspension was passaged three times through a hypodermic needle with 1.2 mm diameter and several times through a needle with 0.8 mm diameter to accomplish complete homogenization of the suspension in order to facilitate

subsequent cell disruption, which was performed via 3 passages through a French press cell at 110 MPa. Subsequently the cell lysate was ultracentrifuged at 125,000 × g for 1 h at 4 °C and the supernatant (cell free extract) was used in the following conversion reaction.

The cell free extract (1.5 mL per assay) was incubated with 33 Bq ^{14}C-labeled fumarate and 100 Bq of either (*R*)- or (*S*)- toluene overnight at room temperature in an anaerobic chamber. This experiment was performed in four independent replicas. The resulting radiolabeled benzylsuccinates were extracted using silica-based C_{18}-columns (Sep-Pak®, Waters). The columns were equilibrated with 0.1% TFA/H_2O, then the samples were loaded and the columns were washed with 1 column volume of 0.1% TFA/H_2O. The benzylsuccinates were eluted using 1 column volume of 0.1% TFA containing 50% acetonitrile. The eluates were dried overnight by evaporating the solvent on a heating plate at 80 °C, and the residues were dissolved in 1.5 mL H_2O. Samples of 500 µL were taken and mixed with 5 mL Quicksafe A scintillation liquid (Zinsser) to determine their radioactivity by scintillation measurements with a PerkinElmer Tri-Carb 2810 TR® Liquid Scintillation Analyzer.

Chemical synthesis of succinyl-CoA and benzylsuccinyl-CoA

Succinic anhydride (7.4 mg, 74 µmol) was dissolved in 1 mL acetonitrile and coenzyme A (50 mg, 61 µmol) was dissolved in 2 mL 1 M $NaHCO_3$. These solutions were mixed, diluted with water to 10 mL and incubated on ice for 1 h. To check the conversion progress, 10 µL portions of the mixture were retrieved and reacted with 10 µL of Ellmann's reagent (20 mg DTNB, 30 mg $KHCO_3$ in 1 mL H_2O) on a filter paper. When the absence of yellow color indicated the completion of succinyl-CoA formation, the reaction was terminated by acidification to pH 1.5 using 5 M aqueous HCl. After degassing of the reaction mixture, succinyl-CoA was purified by solid-phase extraction, using a silica-based C_{18} column as described above. Benzylsuccinyl-CoA was synthesized as described previously.[5] Benzylsuccinate (384 µmol) was dissolved in 800 µL glacial acetic acid at 80 °C and acetic anhydride (384 µmol) was added. The temperature was increased to 120 °C and the reaction was incubated until acetic acid had evaporated completely. This process yielded benzylsuccinic anhydride, which solidified while cooling down. To obtain the thioester, the anhydride was dissolved in 1.5 mL acetonitrile, mixed with coenzyme A (50 mg) dissolved in 2 mL 1 M $NaHCO_3$ and diluted with 7 mL H_2O. The reaction progress was checked using Ellmann's reagent (see above). The reaction was terminated by acidification to pH 1.5 using 5 M HCl. After degassing of the reaction mixture, benzylsuccinyl-CoA was purified by solid-phase extraction, using a silica-based C_{18} column as described above.

Thioester concentrations in the individual fractions were determined by measuring the absorbance at 259 nm (ε = 15.6 M^{-1} cm^{-1}). The eluted fractions were frozen in aliquots of 2 µmol at -80 °C for 30 min, and the remaining acetonitrile was removed via SpeedVac centrifugation until the volume of the aliquots was reduced by one half. The aliquots were frozen another time at -80 °C for overnight lyophilization. The dried aliquots were stored at -80 °C until use.

Purification of succinyl-CoA:benzylsuccinate CoA-transferase (BbsEF) and succinyl-CoA dehydrogenase (BbsG)

We used recombinant succinyl-CoA:benzylsuccinate CoA-transferase (BS-CT) from the toluene-degrading $Fe^{(III)}$ reducing species *Geobacter metallireducens*, because the previously characterized BS-CT orthologue from *T. aromatica* is rather unstable and could not be produced recombinantly.[5] The enzyme (BbsEF subunits; accession numbers WP_011365841 and WP_004511545) containing an N-terminal strep-tag was heterologously overproduced in *E. coli* DH5α in LB-medium. Gene expression was induced with anhydrotetracycline (0.2 µg/mL; 2 mg/mL in the stock solution) when an OD of 0.5 of the culture was reached. The cells were incubated at room temperature for 14 h, centrifuged at 4,000 × g for 10 min, and the cell pellet was re-suspended in 10 mM Tris/Cl buffer (pH 7.5). Cell disruption was performed via sonication followed by a final centrifugation for 1 h at 4 °C at 100,000 × g. The enzyme was purified using affinity chromatography on a streptavidin column. The desired protein was eluted using a 100 mM Tris/Cl buffer (pH 8) (buffer A) containing 2.5 mM desthiobiotin, yielding 1.25 mg of BbsEF per g of wet cell mass.

Benzylsuccinyl-CoA dehydrogenase (BS-DH) from *T. aromatica* (subunit accession number AAF89842) was overproduced in *E. coli* DH5α cells containing the plasmid pTrc99aBbsG_Taro.[5] Overproduction was induced using 1 mM IPTG at an optical density 1 at 578 nm. After incubation for 4 h at 30 °C, the cells were harvested via centrifugation at 6000 × g at 4 °C for 10 min. After overproduction, the cells were re-suspended in 10 mM triethanolamine/NaOH buffer (pH 7.5) containing 10 % glycerol (buffer B). Cell disruption was performed via 3 passages over a French press cell at 110 MPa. Subsequently, the cell lysate was ultracentrifugated at 120,000 × g for 1 h at 4 °C. The supernatant was filtrated through a 0.2 µm pore size filter, obtaining the cell free extract. The extract was applied to a DEAE Sepharose fast flow column, which was washed with 2 column volumes of buffer B and eluted by applying a linear gradient from 0 – 500 mM NaCl in buffer B over 8 column volumes. The

protein eluted at NaCl concentration between 225 – 290 mM. The active fractions exhibited a yellow color and were pooled and concentrated using a Sartorius Stedim Vivaspin 20 cartridge.

Conversion of benzylsuccinate to (*E*)-benzylidenesuccinyl-CoA

Preliminary experiments with unlabeled compounds indicated that activation of the extracted benzylsuccinates to the CoA-thioesters was insufficient when benzylsuccinyl-CoA was synthesized chemically via benzylsuccinic anhydride as described, yielding only 35% conversion of benzylsuccinate to benzylidenesuccinyl-CoA. In contrast, coupled conversion assays with succinyl-CoA and purified recombinant BS-CT and BS-DH, the next two enzymes of the anaerobic toluene metabolic pathway,[5] gave conversion yields of 90% (data not shown). The extracted doubly-labeled benzylsuccinates (1 mL portions in 1 M Tris/HCl buffer, pH 7) were combined with 16 µmol succinyl-CoA dissolved in 6.5 mL of a 200 µM ferricenium hexafluorophosphate solution, 2 mM $MgCl_2$ and 20 mM KCl , diluted with water to a reaction volume of 10 mL. The reaction was started by adding 1 U (250 µL) of BS-CT and 1 U (1 mL) of BS-DH and incubated at room temperature for 3 h. During this time, the color of the assay mixture changed rapidly from green (a mixture of the blue ferricenium ion and the yellow BS-DH) to yellow, indicating the reduction of ferricenium hexafluorophosphate to ferrocene and the simultaneous formation of benzylidenesuccinyl-CoA. After completion, the assay was acidified to pH 1.5 by the stepwise addition of 5 - 20 µL portions of 5 M HCl. After centrifugation, the supernatant was loaded onto a C_{18} solid phase extraction column as described above. Following elution, the eluate was dried overnight by evaporating the solvent on a heating plate (80 °C). The dried eluates were taken up in 1.5 mL water and 500 µL of the resulting solution was mixed with the scintillation liquid as described above to determine ^{3}H and ^{14}C radioactivity by scintillation measurements. Retention or loss of ^{3}H was based on the calculated values of the $^{3}H/^{14}C$ ratios in the samples containing benzylsuccinate and benzylidenesuccinyl-CoA, respectively. Because the actual enantiomeric purity of the synthesized (*R*)- and (*S*)-toluenes could not be determined independently, we calculated the expected rates of ^{3}H retention in benzylidenesuccinyl-CoA based on isotope effects of 4.0 and 7.4 for different enantiomeric purities of the chiral toluenes (table S1). Matching of the experimental data with the expected values suggests enantiomer purities of both enantiomers higher than 90%.

Table S1: Expected retainment of $^{3}H/^{14}C$ ratios in benzylidenesuccinyl-CoA with different enantiomeric purities of chiral toluenes. The numbers are based on isotope effects of 4.0 ($^{2}H/^{1}H$) and 7.4 ($^{3}H/^{1}H$), which are expected to remove the different hydrogen isotopes at 72% (^{1}H), 18% (^{2}H), and 10% (^{3}H).

Toluene enantiomer purity	100%	90%	80%	experimental
$^{3}H/^{14}C$ ratio from (*S*)-toluene	82%	76%	69%	79%
$^{3}H/^{14}C$ ratio from (*R*)-toluene	28%	33%	38%	25%

Theoretical transition state modeling

The initial geometry of the α subunit of BSS was taken from the crystal structure (PDB code: 4PKF).[6a] The β and γ subunits as well as water molecules were removed and protons were added by the programs Calculate Protein Ionisation and Residue pK of Biovia Discovery Studio 4.0, assuming an optimum pH value of 7.4. All calculations in DS 4.0 were conducted using CHARMm force field. [7] After local geometry minimization of the active site residues, the model obtained was used for docking (*R*)-benzylsuccinate in four different protonation states using the Libdock protocol. [8] The best-fitting protonation state of the product was selected based on the lowest binding energy of the respective molecule. The model obtained for an enzyme-product complex was solvated and the geometry was again minimized (using an RMS gradient cut-off at 0.000010 kcal/(mol*Å)), followed by an MD simulation cascade consisting of *in-silico*-"heating" up to 300 K for 10 ps, a model equilibrating phase for 1000 ps and a final MD simulation phase for 5000 ps. The resulting structure exhibiting the lowest total energy was used as initial model for further docking and molecular mechanics studies. Subsequently, the bound product molecule was replaced by the substrates toluene and mono-protonated fumarate, Cys493 was modified to represent a thiyl radical state and the geometry of the whole active site model was again minimized. The structure obtained was used to define a QM cluster containing whole or fragmented side chains of active site amino acids surrounding the bound substrates (i.e. Glu189, Tyr197, Ser199, Ile384, Leu391, Leu492, Cys493, Arg508, and Val709) with added geometry constrains. Cluster model calculations were then conducted in Gaussian 09 (B3LYP functional with D2 Grimme empirical dispersion correction using 6-31g(d,p) basis set for geometry optimization). The transition state for C-H activation was localized by potential energy scans along approximate reaction coordinates, followed by full optimization of the TS geometries using the Berny algorithm.[9]

To obtain kinetic isotope effects, the zero-point energy corrections, obtained at the B3LYP/6-31g(d,p) level, were calculated for a temperature of 303 K for toluene, [$^{2}H_{8}$]-toluene as well as (*S*)- and (*R*)-toluene. The kinetic constants were calculated according to the Equation 1.

$$k_X = \frac{k_B T}{h} e^{\left(-\frac{\Delta(E+ZPE)_X}{RT}\right)}, \text{ Eq. 1}$$

where X stands for either H or ^{2}H. The isotope effect was calculated as the ratio of k_H to k_D or k_H to k_T. An estimation of combined primary and secondary KIE for conversion of unlabelled toluene vs. [$^2H_8$] -toluene yielded a theoretical KIE value of 5.7 with zero-point correction. A similar estimation for H vs. ^{2}H in chirally labelled toluene enantiomers used in this study yielded values of 6.1 for (*R*)- and 6.9 for (*S*)-toluene, respectively. Regarding the limited extent of the active-site amino acids that could be integrated into the model, these predicted KIE values correlate sufficiently well with the experimental value of 4.0 for the deuterium KIE. Therefore, the calculations in this study were based on KIE values of 4.0 and 7.6 for deuterium and tritium, respectively.[9]

The cluster model of the active site of BSS contained all important interactions involved in positioning of the fumarate co-substrate.[9] The most important interaction appears to be a salt bridge between Arg508 and the deprotonated carboxyl group of fumarate, as suggested previously.[6] This is supplemented by additional hydrogen bonds with Ser199, Tyr 197 and Asn615. The other carboxyl group of the fumarate is predicted to be in the non-dissociated form in the model, forming an H-bond with the backbone amide group between Leu492 and Cys493. The second bound substrate, toluene, interacts exclusively via van der Waals interactions with several hydrophobic amino acids lining the cavity walls (e.g. Ile384) and the co-substrate fumarate (Fig. S1). The geometry of the calculated first transition state contains the methyl group of toluene lined up with the thiyl radical group of Cys493. The calculated bond distances of the hydrogen atom to be abstracted towards the substrate C atom and the enzymatic S atom are almost equal (1.49 Å and 1.51 Å) and the S-H-C angle is 163.5^o (Fig. S2), facilitating the transfer. Two thirds of the calculated radical spin density are located on the toluene (54% localized on the methyl carbon), while 33% are still localized on the S atom of Cys493.[9]

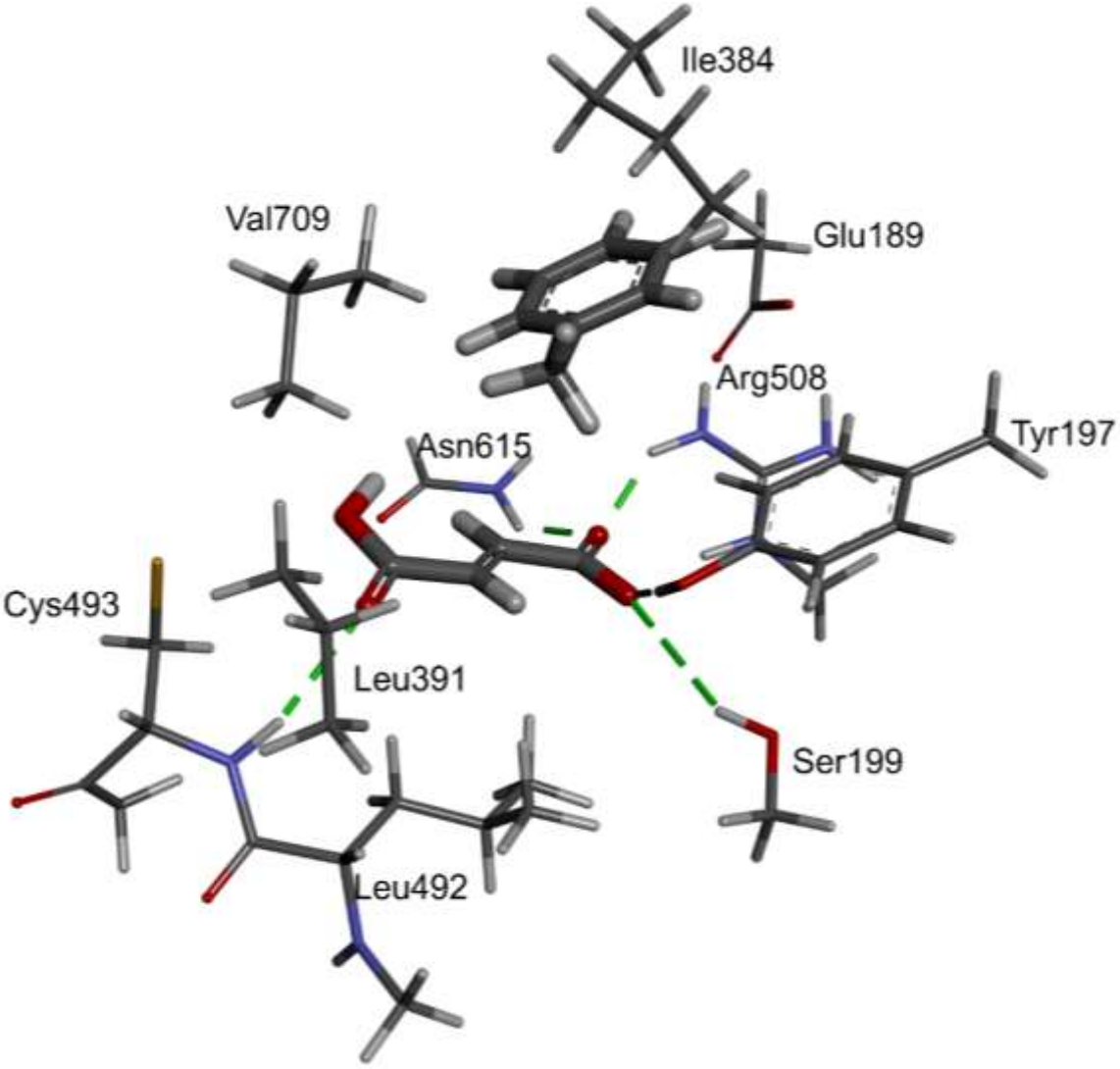


Fig. S1. The structure of ES complex in the cluster model. Green lines depict H-bond interactions.

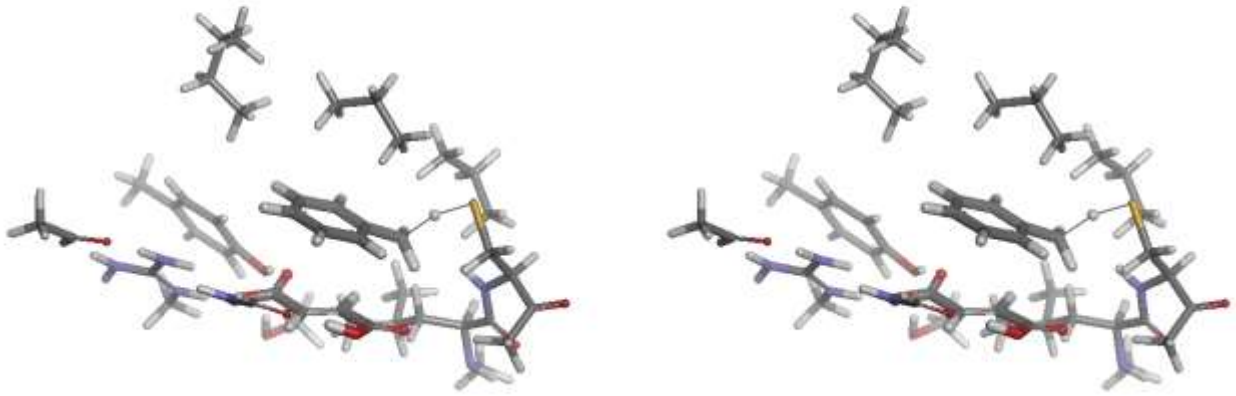


Fig. S2: 3D-view of a model of the active site of BSS containing both substrates (fat lines). The first transition state is shown during the initial hydrogen transfer from toluene to the thiyl radical on Cys493 (dotted lines). The fumarate co-substrate is bound in a conformation that only allows addition to the generated benzyl radical with inversion of its stereochemistry.

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